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Ontogenetic expression of the *Otx2* and *Crx* homeobox genes in the retina of the rat

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Abstract

Otx2 and *Crx* are vertebrate orthologs of the orthodenticle family of homeobox genes, which are involved in retinal development. In this study, the temporal expression patterns of *Otx2* and *Crx* in the rat retina during embryonic and postnatal stages of development were analyzed in detail. This confirmed the presence of Otx2 mRNA in both the embryonic retinal pigment epithelium and the developing neural retina. During development, the expression of *Otx2* persists in the pigment epithelium, whereas *Otx2* expression of the neural retina becomes progressively restricted to the outer nuclear layer and the outer part of the inner nuclear layer. Immunohistochemistry revealed that Otx2 protein is also present in cell bodies of the ganglion cell layer, which does not contain the Otx2 transcript, suggesting that Otx2 protein is synthesized in cell bodies of the bipolar neurons and then transported to and taken up by cells in the ganglion cell layer. *Crx* is also highly expressed in the outer nuclear layer starting at E17 and postnatally in the inner nuclear layer. The onset of expression of *Crx* lags behind that of *Otx2* consistent with evidence that Otx2 activates *Crx* transcription. These expression patterns are consistent with evidence that *Otx2* and *Crx* function during retinal development and extend the period of probable functionality to the adult. In this regard, these results provide an enhanced and expanded temporal and spatial framework for understanding the multiple roles of *Otx2* and *Crx* in the developing and mature mammalian retina.

Keywords: Otx2; Crx; homeobox; retina; development; rat

1. Introduction

Homeobox genes encode transcription factors that regulate morphogenesis and differentiation processes in all eukaryotes. The orthodenticle group of homeobox genes plays a highly conserved role in development of rostral brain regions and sensory organs including eyes and photoreceptors (reviewed by Arendt, 2003). The *Otx2* and cone–rod homeobox (*Crx*) genes are vertebrate members of the orthodenticle group (Simeone et al., 1992, 1993; Chen et al., 1997; Furukawa et al., 1997). *Otx2* plays a crucial role in general development of the vertebrate eye as evidenced by studies on *Otx2* deficient mice (Matsuo et al., 1995; Martinez-Morales et al., 2001) and ectopic expression experiments (Zuber et al., 2003). In the retina, *Otx2* has been shown to be involved in differentiation of the retinal pigment epithelium (Martinez-Morales et al., 2003), bipolar cells (Viczian et al., 2003) and photoreceptors (Nishida et al., 2003; Akagi et al., 2004). Contrarily, the role of *Crx* in retinal development seems to be limited to differentiation of photoreceptor cells (Furukawa et al.,

Abbreviations: AANAT, arylalkylamine *N*-acetyltransferase; Crx, cone–rod homeobox; GCL, ganglion cell layer; HIOMT, hydroxyindole-*O*-methyltransferase; INL, inner nuclear layer; IPL, inner plexiform layer; NR, neural retina; ONL, outer nuclear layer; OPL, outer plexiform layer; RPE, retinal pigment epithelium; SCN, suprachiasmatic nucleus.

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1999). Interestingly, *Otx2* has been shown to be essential for transactivation of *Crx* (Nishida et al., 2003), which in turn induces expression of photoreceptor-specific genes involved in phototransduction (Chen et al., 1997; Livesey et al., 2000).

Otx2 and *Crx* are also recognized as playing a role in the pineal gland (Furukawa et al., 1999; Nishida et al., 2003; Rath et al., 2006). This is consistent with the view that both the pineal gland and the retinal photoreceptor evolved from a common ancestral photodetector (reviewed by Klein, 2004, 2006). Recent observations have raised the possibility that these transcription factors, in addition to playing a role in development, are also important in maintaining phenotype of these cells, because *Otx2* and *Crx* are expressed in the adult pineal gland (Rath et al., 2006).

Although it is clear that *Otx2* and *Crx* are expressed in the retina, a detailed spatial and temporal analysis of the expression of these transcription factors is not available. This was accomplished in the present study, in which the expression of *Otx2* and *Crx* in the rat retina was studied during development into adulthood. As presented here, it is clear that both genes are not only expressed during early developmental stages, but are also expressed in the adult, which supports the view that they function not only to initiate developmental expression of genes in the retina, but also to maintain the molecular phenotype. This may not only involve classical mechanisms of homeodomain control of gene expression. Our observations suggest that a novel cell-to-cell transfer mechanism might also be involved, because Otx2 protein was found in ganglion cells, which do not appear to express the *Otx2* gene.

2. Materials and methods

2.1. Animals

Fetal and postnatal Sprague–Dawley rats (Charles River, Sulzfeld, Germany) were sacrificed during daytime. In the embryonic series (E15–E21), the timed-pregnant mothers were anesthetized with tribromethanol (500 mg/kg). A superior–inferior section was done in the midline of the abdomen and the uterus was opened. The fetuses were removed and the heads were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The postnatal animals were decapitated and the heads (P2–P6) or the eyes (P12–P30) were fixed by immersion in the same fixative. Immersion fixation was performed at 4 °C for 2 days. After fixation, the tissues were cryoprotected in 25% sucrose and frozen on crushed carbon dioxide.

For unilateral lesion of the optic nerve male Sprague–Dawley rats, weighing 180–200 g, were anesthetized with tribromethanol (500 mg/kg). A section was done in the lateral part of the orbit and the optic nerve was cut. The animals were perfusion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) 3, 7 or 28 days after surgery. After fixation, the tissues were cryoprotected in 25% sucrose and frozen on crushed carbon dioxide. For western blotting, male Sprague–Dawley rats, weighing 200–250 g, were anesthetized with tribromethanol and sacrificed by decapitation. All experiments with animals were performed in accordance with the guidelines of EU *Directive 86/609/EEC* approved by the Danish Council for Animal Experiments.

2.2. In situ hybridization

Cryostat sections, 14 μm in thickness, were cut and mounted on Superfrost Plus® slides (Menzel, Braunschweig, Germany). Tissue sections were thawed and washed 2 × 1 min in PBS. This was followed by acetylation in 0.25% acetic anhydride (diluted in 0.1 M triethanolamine and 0.9% NaCl) for 10 min. The sections were then dehydrated in a graded series of ethanol and delipidated in 100% chloroform, followed by partial rehydration in 95% ethanol. For hybridization, the following 38-mer DNA probes were used.

(1) 5′-CGAGCCAGCATAGCCTTGACTATAACCTGAAGC CTGAG-3′; antisense, position 910–873 on rat Otx2 mRNA (XM224009.3).
(2) 5′-CTCAGGCTTCAGGTTATAGTCAAGGCTATGCTGG CTCG-3′; sense, position 873–910 on rat Otx2 mRNA (XM224009.3).
(3) 5′-AAGTTGTGCCCTCGTGAAAGTAGTCCTCTCCCT CCGCT-3′; antisense, position 484–447 on rat Otx2 mRNA (XM224009.3).
(4) 5′-AGCGGAGGGAGAGGACTACTTTCACGAGGGCA CAACTT-3′; sense, position 447–484 on rat Otx2 mRNA (XM224009.3).
(5) 5′-GATCTTGAGAGCAACCTCCTCACGTGCATACAC ATCCG-3′; antisense, position 228–191 on rat Crx mRNA (NM021855.1).
(6) 5′-CGGATGTGTATGCACGTGAGGAGGTTGCTCTCA AGATC-3′; sense, position 191–228 on rat Crx mRNA (NM021855.1).

Probes were diluted in DEPC-treated water to a concentration of 5 pmol/μl. Five microlitres of the probe was then labeled with [^{35}S]dATP by use of terminal transferase (Roche, Penzberg, Germany) to a specific activity of 1×10^{18} dpm/mol. The labeled probe was diluted in hybridization buffer (10 μl labeled probe/ml hybridization buffer) consisting of 50% (v/v) formamide, 4× SSC (SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 1× Denhardt solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% ficoll), 10% (w/v) dextran sulfate, 10 mM dithiothreitol, 0.5 mg/ml salmon sperm DNA and 0.5 mg/ml yeast tRNA. The sections were hybridized in a humid chamber overnight at 37 °C. After hybridization, the slides were washed in 1× SSC for 4 × 15 min at 55 °C, 2 × 30 min at room temperature, and rinsed in deionized water.

The sections were dried and exposed to an X-ray film for 1–2 weeks. The images of the sections on the X-ray film were transferred to a computer and quantified (Image 1.42 software, Wayne Rasband, National Institutes of Health, Bethesda, MD). Optical densities were converted to dpm/mg tissue by using simultaneously exposed ^{14}C-standards calibrated by comparison with ^{35}S-tissue paste standards. The

sections were subsequently dipped in a photographic emulsion (Amersham, Hillerød, Denmark) and exposed for 2–3 weeks. After exposure, the autoradiographs were developed in amidol and fixed in thiosulfate. The sections were counterstained in cresyl violet and photographed in a microscope either in dark field mode or in transmitted light.

2.3. Immunohistochemistry

Cryostat sections, 14 μm in thickness, were cut and mounted on Superfrost Plus® slides (Menzel, Braunschweig, Germany). The sections were washed in PBS for 3 × 5 min and pre-incubated in 5% normal swine serum diluted in PBS for 30 min. This was followed by incubation overnight in 10 μg/ml immunopurified polyclonal goat anti-human OTX2 IgG (R&D Systems, Abingdon, UK, #AF1979) in PBS with 1% bovine serum albumin and 0.3% Triton X-100 at 4 °C. Alternatively, sections were incubated overnight in immunopurified polyclonal goat anti-human OTX2 (P-15) IgG (Santa Cruz Biotechnology, Santa Cruz, CA, #sc-30659) diluted 1/200 in PBS with 1% bovine serum albumin and 0.3% Triton X-100 at 4 °C. For control of immunospecificity, the diluted polyclonal goat anti-human OTX2 (P-15) IgG (Santa Cruz Biotechnology, Santa Cruz, CA, #sc-30659) was pre-absorbed for 72 h at 4 °C by use of the OTX2 epitope (Santa Cruz Biotechnology, Santa Cruz, CA, #sc-30659P) in a concentration of 100 μg/ml. After incubation in primary anti-sera, the sections were washed 3 × 10 min in PBS added 0.25% bovine serum albumin and 0.1% Triton X-100 followed by incubation for 1 h in biotinylated donkey anti-goat IgG (Jackson Immunoresearch, Soham, UK) diluted 1:500 in the same buffer. The sections were washed 3 × 5 min in PBS with 0.1% Triton X-100 and incubated for 45 min in ABC-Vectastain solution (Vector Laboratories, Burlingame, CA) diluted 1:100 in the same buffer. After washing 3 × 5 min in PBS with 0.1% Triton X-100, the sections were incubated in biotinylated tyramide diluted 1:100 in PBS containing 0.005% H_2O_2, then washed 3 × 5 min in PBS with 0.1% Triton X-100, and incubated for 30 min in ABC-Vectastain solution diluted 1:100. After washing for 2 × 5 min in PBS with 0.1% Triton X-100 and 10 min in 0.05 M Tris (pH 7.6), the sections were incubated for peroxidase activity in 1.4 mM diaminobenzidine (Sigma, Steinheim, Germany) and 0.01% H_2O_2 in 0.05 M Tris (pH 7.6) for 15 min. The reaction was terminated by washing the sections in excessive amounts of deionized water. Finally, the sections were dried and embedded in Pertex® (Histolab, Gothenburg, Sweden).

2.4. Western blot analysis

Samples were obtained from pools of tissues from five animals. Samples were homogenized in a 2× Laemmli buffer containing 73% 155 mM Tris buffer (pH 8.3), 9% SDS, 16 mM bromphenol blue, 18% glycerol and 10% 2-mercaptoethanol (0.1 g tissue/ml buffer). Samples were boiled and centrifuged at 13 000 × *g* for 1 h at 4 °C. Protein content of the supernatants was determined by use of the RC DC Protein Assay (BioRad, Hercules, CA). 50, 100 or 150 μg of protein per lane was run in a NuPAGE® Bis-Tris 12% Gel and transferred to a nitrocellulose membrane by use of the XCell® Surelock Mini-Cell system (Invitrogen, Taastrup, Denmark). The membrane was blocked in blocking solution (Amersham, Hillerød, Denmark). The membrane was incubated in 4 μg/ml polyclonal goat anti-human OTX2 IgG (R&D Systems, Abingdon, UK) diluted in blocking solution for 1 h. The membrane was washed in PBS and subsequently incubated in biotinylated donkey anti-goat IgG (Jackson Immunoresearch, Soham, UK) diluted 1:500 in blocking solution for 1 h and washed in PBS. The membrane was incubated in ABC-Vectastain solution (Vector Laboratories, Burlingame, CA) diluted 1:100 in blocking solution and subsequently washed in PBS and 0.05 M Tris (pH 7.6). Chromogenic development was done by incubating the membrane in 1.4 mM diaminobenzidine (Sigma, Steinheim, Germany) and 0.01% H_2O_2 in 0.05 M Tris (pH 7.6); the reaction was stopped by washing in deionized water. Protein size was estimated by comparison with high-range molecular weight markers (Amersham, Hillerød, Denmark).

3. Results

3.1. Ontogenetic Otx2 expression in the rat retina

The ontogenetic expression pattern of *Otx2* in the rat retina was monitored using in situ hybridization of sections of the eyes of animals sacrificed at 11 stages (E16–P30; Fig. 1A–J; Table 1). Otx2 mRNA was detected at all stages examined. At E16, a strong signal was observed in the retinal pigment epithelium; a moderate signal was seen in the neural retina, in which the intensity decreased along a gradient towards the inner part (Fig. 1A, F). During the following embryonic days, the signal of the pigment epithelium declined, whereas the signal in the neural retina increased. From E19 to E21, the labeling density of the outer part of the neural retina and the retinal pigment epithelium was of equal intensity (Fig. 1B, G). In contrast, a signal was not detected in the developing ganglion cell layer. At P2 and P6, a very strong signal was observed in the outer part of the neural retina corresponding to the presumptive outer nuclear layer and the outer part of the inner nuclear layer. The signal of the pigment epithelium was detectable above background, whereas a specific signal was not observed in the ganglion cell layer (Fig. 1C, H). At P12 and P18, the Otx2 transcript was detected in the inner segments of the photoreceptors and in the outer nuclear layer, but the strongest signal was observed in outer part of the inner nuclear layer (Fig. 1D, I). At this stage, a weak signal of the pigment epithelium was detectable. This spatial pattern of *Otx2* expression persisted in the adult, albeit at a somewhat lower intensity. This distribution of Otx2 mRNA was detected with two different probes and the specificity of the signal was confirmed by use of sense control probes (Fig. 1E,J).

Otx2 protein was studied in a similar developmental series (Fig. 1K–O; Table 1). At E16, strong nuclear Otx2 immunoreactivity was observed in all cells of the retinal pigment

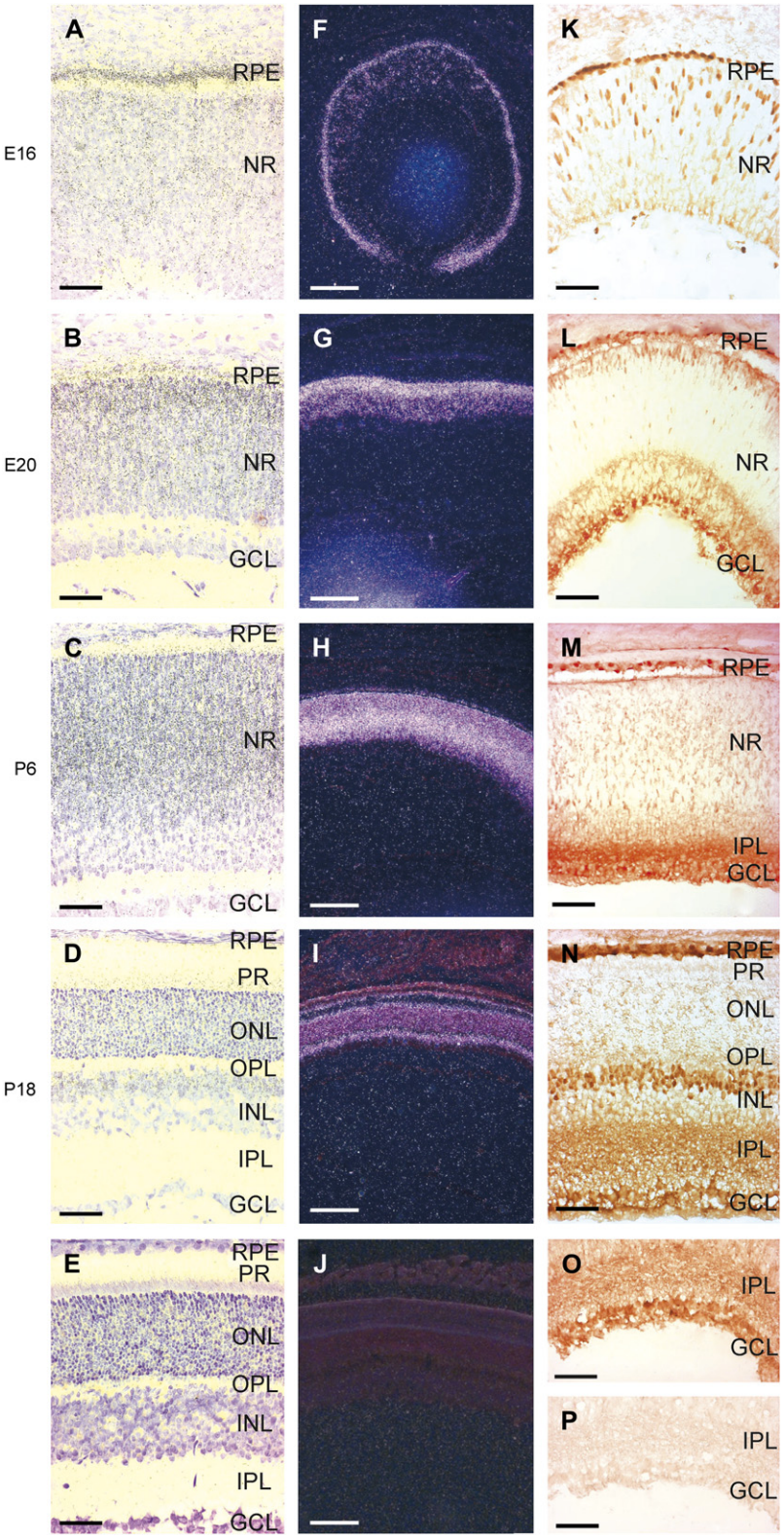

Fig. 1. *Otx2* expression in the developing rat retina. A–D: radioactive in situ hybridization for detection of Otx2 mRNA in the retina of the rat at E16 (A), E20 (B), P6 (C) and P18 (D). Scale bar: 50 μm. E: retinal section (P18) hybridized with a sense control probe. Scale bar: 50 μm. F–I: dark field images of radioactive in situ hybridization for detection of Otx2 mRNA in the retina of the rat at E16 (F), E20 (G), P6 (H) and P18 (I). Scale bar: 200 μm. J: dark field image of a retinal section (P18) hybridized with a sense control probe. Scale bar: 200 μm. K–L: immunohistochemical detection of Otx2 protein in the rat retina at E16 (K), E20 (L), P6 (M) and P18 (N) by use of polyclonal goat anti-human OTX2 IgG (R&D Systems). Scale bar: 50 μm. O: immunohistochemical detection of Otx2 protein in inner part of the rat retina (P18) by use of polyclonal goat anti-human OTX2 (P-15) IgG (Santa Cruz Biotechnology). Scale bar: 50 μm. P: immunohistochemical pre-absorption control (P18). Scale bar: 50 μm. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; NR, neural retina; ONL, outer nuclear layer; OPL, outer plexiform layer; PR, photoreceptors; RPE, retinal pigment epithelium.

Table 1
Expression of Otx2 mRNA/protein and Crx mRNA in distinct layers of the developing rat retina based on in situ hybridization and immunohistochemical results

				E16	E17–E21	P2–P6	P12	P18	P30
Otx2	mRNA	RPE		+++	++	+	+	+	+
		Neural retina	ONL	+	++[a]	+++[a]	++	+	+
			INL				+++[a]	++[a]	++[a]
			GCL		–	–	–	–	–
	Protein	RPE		+++	+++	++	++	++	++
		Neural retina	ONL	+	+[a]	+[a]	+	+	+
			INL				++[a]	++[a]	++[a]
			GCL		++	++	+++	++	++
Crx	mRNA	RPE		–	–	–	–	–	–
		Neural retina	ONL	–	+[a]	++[a]	+++	+++	++
			INL				+	+	–
			GCL		–	–	–	–	–

Expression levels in each identifiable retinal layer were visually scored: –, no signal above background; +, weak signal; ++, moderate signal; +++, strong signal. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium.
[a] Score applies to outer part of retinal layer.

epithelium and in a subset of cells in the neural retina; the highest number in this subset was in the outer region (Fig. 1K). A similar distribution of Otx2 immunoreactivity was detected in the outer part of the retina at later embryonic stages (Fig. 1L). Postnatally, Otx2 immunoreactivity was detected in the retinal pigment epithelium; however, nuclear staining in the neural retina was confined to cells in the outer part of the inner nuclear layer, whereas a weaker cytoplasmic signal was present in the outer nuclear layer (Fig. 1M, N).

Further, a strong non-nuclear cytoplasmic staining was seen in the inner plexiform layer and ganglion cell layer with a weaker cytoplasmic staining of the outer nuclear layer (Fig. 1L, M, N). Otx2 immunostaining of the inner plexiform layer and ganglion cell layer was detected with two different anti-sera (Fig. 1N, O); specificity of the staining was confirmed by a pre-absorption control (Fig. 1P).

3.2. Distribution of Otx2 protein in the retinohypothalamic tract

The finding of Otx2 immunoreactivity in retinal ganglion cells prompted us to investigate a possible anterograde transport of Otx2 protein from the retina to the suprachiasmatic nucleus (SCN) via the retinohypothalamic tract, which could explain our previous finding that Otx2 protein is present in the SCN of the rat (Rath et al., 2006). However, western blot analysis failed to reveal the presence of Otx2 protein in the optic nerve (Fig. 2). In addition, lesioning of the optic nerve did not alter the presence or distribution of Otx2-immunoreactivity within the SCN at 3, 7 or 28 days after surgery (data not shown).

3.3. Ontogenetic Crx expression in the rat retina

Analysis of the retinal developmental expression pattern of *Crx* (Fig. 3; Table 1) indicated that it was not expressed at detectable levels at E15 and E16. However, *Crx* expression was detected at E17 in the outer part of the neural retina (Fig. 3A, F). A similar spatial expression pattern was observed until birth (Fig. 3B, G). In the postnatal retina, a strong Crx signal was detected in the inner segments of the photoreceptors and in the outer nuclear layer, whereas a weaker signal was seen in the inner nuclear layer (Fig. 3C, H). At P30, the Crx transcript was present in the inner segments of the photoreceptors and in the outer nuclear layer; however, at this time, a Crx signal was not detected in the inner nuclear layer (Fig. 3D, I). The specificity of the signal was confirmed by use of a sense control probe (Fig. 3E, J).

3.4. Densitometric quantification of the Otx2 and Crx in situ signals on X-ray film

Densitometric quantification of total retinal Otx2 mRNA revealed an expression profile with increasing Otx2 mRNA levels during late embryonic and early postnatal stages and a peak at P6 followed by a decreasing, but sustained retinal *Otx2* expression (Fig. 4). The expression of *Crx* in the retina

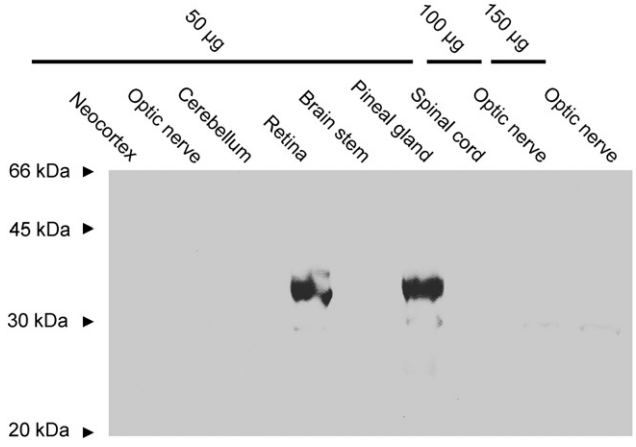

Fig. 2. Western blot analysis of the presence of Otx2 protein in the central nervous system of the rat. The predicted sequence of the rat Otx2 protein (XP2204009) has a molecular weight of 31.6 kDa. The amount of protein loaded in each lane (50, 100 and 150 µg, respectively) is given above the horizontal lines. Arrows indicate molecular weights established by running a standard molecular weight marker.

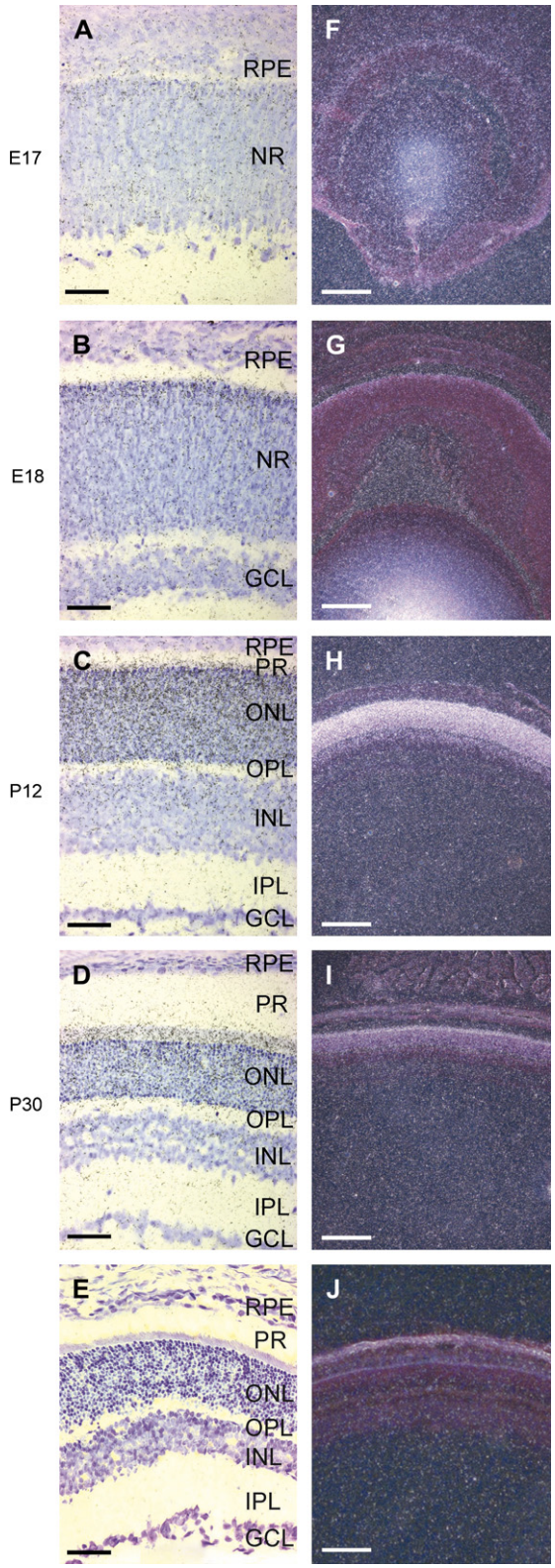

Fig. 3. *Crx* expression in the developing rat retina. A–D: radioactive in situ hybridization for detection of Crx mRNA in the retina of the rat at E17 (A), E21 (B), P12 (C) and P30 (D). Scale bar: 50 μm. E: retinal section (P30) hybridized with a sense control probe. Scale bar: 50 μm. F–I: dark field images of radioactive in situ hybridization for detection of Crx mRNA in the retina of the rat at E17 (F), E21 (G), P12 (H) and P30 (I). Scale bar: 200 μm. J: dark field image of a retinal section (P30) hybridized with a sense control probe. Scale bar: 200 μm. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; NR, neural retina; ONL, outer nuclear layer; OPL, outer plexiform layer; PR, photoreceptors; RPE, retinal pigment epithelium.

was also found to increase from the first detectable level at E17 towards a peak around P12 before stabilizing at a lower level in the adult retina.

4. Discussion

The results presented here provide a detailed analysis of the expression of *Otx2* and *Crx* in the developing rat retina. Both *Otx2* and *Crx* are involved in differentiation of various cell types of the vertebrate retina, and the ability of Otx2 and Crx to activate genes involved in phototransduction as well as melanin and melatonin synthesis place these two transcription factors with an important role also in adult retinal physiology. The present study provides a temporal and spatial framework, which is necessary for a detailed mapping and understanding of the multiple roles of *Otx2* and *Crx* in the mammalian retina.

Our results on the rat retina show a high *Otx2* expression in the retinal pigment epithelium in the earlier developmental stages. This early expression in cells of the pigment epithelium is also observed in the mouse (Martinez-Morales et al., 2001; Nishida et al., 2003) and chicken (Bovolenta et al., 1997). In addition, our study revealed a sustained expression of *Otx2* in the mature retinal pigment epithelium of the rat. Functionally, the expression of *Otx2* in the developing pigment epithelial cells is important for the differentiation of this retinal layer as indicated by the findings that *Otx2*-deficient mice fail to properly develop retinal pigment epithelium (Martinez-Morales et al., 2001) and that overexpression of *Otx2* in cultured avian neural stem cells induces a pigmented phenotype (Martinez-Morales et al., 2003). Further, Otx2 binds and transactivates the promoters of several genes encoding melanosome-associated proteins including the enzymes directly involved in melanin biosynthesis (Takeda et al., 2003; Martinez-Morales et al., 2003); the persistent expression of *Otx2* in the retinal pigment epithelium of the adult supports this regulatory function in vivo.

In addition to the pigment epithelium, *Otx2* is also expressed in the neural retina of the rat. Our detection of the Otx2 transcript in retinal photoreceptors as well as cells of the outer part of the inner nuclear layer are in accordance with previous in situ hybridization studies on the retina of the mouse (Nishida et al., 2003; Martinez-Morales et al., 2001). *Otx2* expression is essential for photoreceptor cell fate determination; thus, ablation of *Otx2* in developing photoreceptors of the mouse retina results in differentiation into an amacrine-like cell type (Nishida et al., 2003). Similarly, ectopic expression of *Otx2* induces a photoreceptor-specific phenotype in both cultured mouse retinal progenitor cells (Nishida et al., 2003) and cells from rat iris and ciliary tissue (Akagi et al., 2004). Based on the sustained expression of *Otx2* in the mature neural retina, it also appears reasonable to suspect

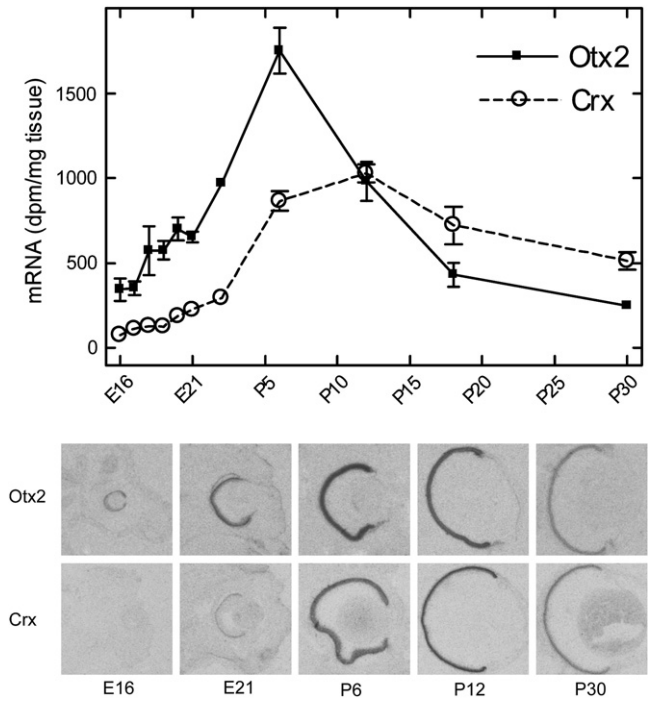

Fig. 4. Densitrometric quantification of Otx2 and Crx mRNA in the developing rat retina. Values on graph represent the mean of at least three animals at each developmental stage examined (E16, E17, E18, E19, E20, E21, P2, P6, P12, P18 and P30). Bars indicate one standard deviation. Lower panel displays representative autoradiographs of retinas hybridized for detection of Otx2 (upper) and Crx (lower) mRNA at indicated developmental stages (E16, E21, P6, P12 and P30).

that this gene plays a role in controlling retinal cell phenotype throughout life.

Translation of the Otx2 mRNA was confirmed in the retinal layers containing the transcript, and our results are in accord with the subcellular distribution of Otx2 in the mouse neural retina with localization in the cytoplasm of photoreceptors and the nuclei of bipolar cells (Baas et al., 2000); however, cytoplasmic Otx2 protein was also found in high amounts in the inner plexiform layer and the ganglion cell layer at all stages examined.

The observation that Otx2 protein is present in the cytoplasm of neurons of the ganglion cell layer, which do not contain the Otx2 transcript, points to the possibility of Otx2 protein being transferred from cells in the outer part of the retina to postsynaptic ganglion cells. Intercellular transport of Otx2 protein, which has been previously described in other systems (Prochiantz and Joliot, 2003), and the binding of Otx2 to translation initiation factor eIF4E (Nedelec et al., 2004), indicate that this transcription factor could act as an intercellular signaling protein and modulate translation in ganglion cells. Notably, Otx2 protein is present in many structures of the rat visual system including the SCN (Nothias et al., 1998; Rath et al., 2006), which is the target nucleus of the retinohypothalamic tract; however, in the present study we were unable to detect a transport of Otx2 protein in the retinohypothalamic tract via optic nerve fibers or to demonstrate that sectioning of the optic nerve altered Otx2 protein in the SCN. Accordingly, it is not possible to claim that Otx2 in the SCN is derived from retinal cells.

Crx is essential for proper development and function of retinal photoreceptors (Furukawa et al., 1999). In the retina of the mouse, *Crx* expression has been reported as early as E12.5 (Furukawa et al., 1997; Nishida et al., 2003). In the present study of the rat, Crx transcripts were not detected before E17, which may be partly explained by the general time lag in rat ontogeny as compared to mouse. Notably, a transient postnatal expression of *Crx* in the inner nuclear layer coincides with the high expression of *Otx2* in the same layer in this period and therefore supports Otx2 as a transcriptional activator of *Crx* (Nishida et al., 2003); however, other factors suggestively also influence *Crx* expression, as Crx transcripts are not detected in the retinal pigment epithelium despite high expression of *Otx2*.

Our quantitative analysis of total *Otx2* and *Crx* expression in the developing retina provides further reason to support the view that Otx2 is a transcriptional activator of *Crx* (Nishida et al., 2003) in vivo, because retinal expression of *Otx2* starts earlier and peaks before expression of *Crx*. This temporal expression pattern is delayed as compared to the expression of these genes in the rat pineal gland, in which a peak in mRNA levels appears just before birth (Rath et al., 2006). Interestingly, the expression of phototransduction genes in the developing murine retina and pineal gland also exhibit a differential temporal expression pattern with high expression levels of these genes in the perinatal pineal gland preceding equivalent postnatal expression levels in the retina (Babila et al., 1992; Blackshaw and Snyder, 1997). In addition to these molecular findings, morphological studies also indicate an earlier maturation of cells of the rodent pineal gland as compared to the retina (Zimmerman and Tso, 1975). In the rat retina, the number of postmitotic cells increases dramatically during the first postnatal week (Alexiades and Cepko, 1996; Livesey and Cepko, 2001). This trend correlates with the postnatal increase in retinal expression of *Otx2* and *Crx*, which are both reported to be expressed postmitotically (Bovolenta et al., 1997; Baas et al., 2000; Garelli et al., 2006). A recent study on ontogenetic *Otx2* expression in the retina and pineal gland of the chicken demonstrated a prenatal rise and peak in Otx2 mRNA levels in both tissues (Dinet et al., 2006); however, these interspecies differences seem to reflect temporal differences in maturation of the avian retina as compared to the rodent (Cepko et al., 1996).

As with pinealocytes, the retinal photoreceptors of most vertebrates also synthesize melatonin from serotonin by the sequential action of the enzymes arylalkylamine *N*-acetyltransferase (AANAT) and hydroxyindole-*O*-methyltransferase (HIOMT) (reviewed by Tosini and Fukuhara, 2003; Iuvone et al., 2005; Klein, 2007). In the eye, melatonin is important for the dark adaptation of the retina by binding Mel_{1c} melatonin receptors on the photoreceptor cells (Wiechmann et al., 2003). Both Otx2 and Crx can bind and transactivate the chicken HIOMT promoter (Dinet et al., 2006; Bernard et al., 2001), and in zebrafish, the expression of AANAT is also regulated by Otx-proteins (Appelbaum et al., 2004, 2005). In

either case, the expression is controlled by highly conserved Otx-binding motifs, which are also found in the mammalian promoters of AANAT and HIOMT (Li et al., 1998). Thus, retinal expression of *Otx2* and *Crx*, in addition to cell fate determination and activation of genes directly involved in phototransduction (Nishida et al., 2003; Furukawa et al., 1997; Chen et al., 1997; Livesey et al., 2000) and melanin synthesis (Takeda et al., 2003; Martinez-Morales et al., 2003), also seems to play a role in melatonin synthesis and dark adaptation in this tissue.

In conclusion, our results establish that *Otx2* and *Crx* are differentially expressed in the developing and mature retina of the rat. The developmental data support the view that both genes are involved in development and postnatal maintenance of retinal cell phenotype. Further, the presence of Otx2 protein in retinal ganglion cells that do not synthesize the Otx2 transcript supports the concept of an intercellular transport of this transcription factor, which might act as a signal protein within the retina.

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